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13. ABSTRACT (Maximum 200 Words)

This project focuses on the development of microfluidic chambers for the study of breast cancer cell chemotaxis. In the first phase of the project, we designed, fabricated, and tested the microfluidic chemotaxis chamber, ensuring its compatibility with breast cancer cell culture and observation using fluorescence and DIC microscopy. We began the next phase of the project, investigating the migration of breast cancer cells in epidermal growth factor (EGF). We showed that the concentration gradients of EGF induce the chemotaxis of breast cancer cells in a manner that is dependent on the shape of the concentration gradient profile. As we continue our work in this project, we will use the microfluidic chemotaxis chamber to study, in depth, the chemotaxis of breast cancer cells in superimposed gradients of multiple growth factors.

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Introduction

This report describes our accomplishments so far in the development microfluidic devices for the study of breast cancer cell migration. Work has initially focused on the design and fabrication of the microfluidic chemotaxis chamber such that it is compatible with breast cancer cell culture and migration. We then began investigating the migration of breast cancer cells in EGF gradients.

Body

The initial phase of the project focused on the development of the microfluidic chemotaxis chamber for the investigation of breast cancer cell chemotaxis. Different designs of the microfluidic network were developed to produce linear and polynomial concentration gradient profiles of epidermal gradient factor (EGF). These gradients were visualized using fluorescence microscopy. The gradients could be maintained as long as flow can be sustained, being limited only by the volumes of growth factor solutions available, thus allowing long term experiments to be performed. To allow culture of cancer cells inside the chambers, the chambers were coated extracellular matrix proteins (ECM) proteins, particularly collagen type IV. The cells attached inside the chamber and were healthy for hours, as seen in their morphology and activity using differential interference contrast (DIC) microscopy. The microfluidic chemotaxis chamber was thus proven to be compatible with MDA-MB-231 cell culture and with both fluorescence and DIC microscopy. Please see attached paper (Wang et al. 2004) for more details.

We then began the next phase of the project, investigating the migration of MDA-MB-231 cells in EGF. Uniform concentrations of EGF resulted in increased motility of cells, but motility was random. We then tested the migration of cells in linear concentration gradients of EGF, but motility was still random, regardless of the slope of the gradient tested. Polynomial EGF gradients, however, induced significant directional migration towards increasing EGF concentration. This demonstrated that the shape of the gradient profile, in addition to the slope, is an important determinant of the resulting migratory response of the breast cancer cells (Wang et al. 2004).

Key Research Accomplishments

- Demonstrated spatial and temporal gradients of chemoattractants
- Optimized the chemotaxis chamber for compatibility with breast cancer cells and capability of long term culture
- Demonstrated the compatibility of the chemotaxis chamber with optical microscopy
- Investigated the migration of the human metastatic breast cancer cell line MDA-MB-231 in linear and polynomial concentration gradients of epidermal growth factor (EGF)
    - Showed that linear gradients of EGF do fail to induce chemotaxis, resulting in random migration of MDA-MB-231 cells
    - Showed that polynomial gradients of EGF induce chemotaxis of MDA-MB-231 cells

Reportable Outcomes

- Journal Article:

  Wang S-J, Saadi W, Lin F, Nguyen CM-C, Jeon NL. Differential effects of EGF gradient profiles on MDA-MB-231 breast cancer cell chemotaxis. *Exp Cell Res* 2004;300:180-89. (Attached in Appendix)

Conclusions

We have developed a microfluidic chamber for the study of breast cancer cell chemotaxis. We optimized the device for the culture and observation of the human metastatic breast cancer cell line MDA-MB-231, and demonstrated its application as an assay of migration for this cell line. Using this assay, we showed that EGF induced chemotaxis depends on the shape o the gradient EGF profile. These findings pave the way to in-depth studies of breast cancer cell chemotaxis. We will be able to compare the effect of different growth factors and other chemoattractants as well as effect of different inhibitors of EGFR or other related growth factor receptors.

References

Wang S-J, Saadi W, Lin F, Nguyen CM-C, Jeon NL. Differential effects of EGF gradient profiles on MDA-MB-231 breast cancer cell chemotaxis. *Exp Cell Res* 2004;300:180-89. (Attached in Appendix)

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Differential effects of EGF gradient profiles on MDA-MB-231 breast cancer cell chemotaxis

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Abstract

Chemotaxis, directed cell migration in a gradient of chemoattractant, is an important biological phenomenon that plays pivotal roles in cancer metastasis. Newly developed microfluidic chemotaxis chambers (MCC) were used to study chemotaxis of metastatic breast cancer cells, MDA-MB-231, in EGF gradients of well-defined profiles. Migration behaviors of MDA-MB-231 cells in uniform concentrations of EGF (0, 25, 50, and 100 ng/ml) and EGF (0–25, 0–50, and 0–100 ng/ml) with linear and nonlinear polynomial profiles were investigated. MDA-MB-231 cells exhibited increased speed and directionality upon stimulation with uniform concentrations of EGF. The cells were viable and motile for over 24 h, confirming the compatibility of MCC with cancer cells. Linear concentration gradients of different ranges were not effective in inducing chemotactic movement as compared to nonlinear gradients. MDA-MB-231 cells migrating in EGF gradient of 0–50 ng/ml nonlinear polynomial profile exhibited marked directional movement toward higher EGF concentration. This result suggests that MDA-MB-231 cancer cell chemotaxis depends on the shape of gradient profile as well as on the range of EGF concentrations.

Keywords: Breast cancer; Chemotaxis; EGF; Migration; Gradient profile; Microfluidics

Introduction

Metastasis is a major cause of treatment failure in cancer patients and a main impediment to improving prognosis [1–3]. Metastasis is a multi-step process that starts when several cells in the primary tumor acquire an invasive phenotype, detach from the original site, pass through blood and lymphatic circulation, and finally settle down and proliferate in secondary organs [3,4]. It has been proposed that metastatic cancer cells are directed to their target organs by soluble factors released by the target organs [1,4–6]. This directed migration or chemotaxis of cancer cells may play an important role during both intravasation and extravasation and has been proposed as one of the crucial steps in cancer metastasis [7].

Growth factors, chemokines, and extracellular matrix components have been reported to play crucial roles in tumor chemotaxis and thus metastasis. Epidermal growth factor (EGF) is an important growth factor associated with the development and spread of breast cancer [8]. In addition to its mitogenic effects, EGF has been shown to be motogenic to breast cancer cells [9–11]. Uniform concentrations of EGF induce chemokinesis, while EGF gradients induce chemotaxis [12–14]. In a series of thorough studies, Segall et al. [17] have shown that EGF gradients generated with a micropipette specifically induced polarized morphology and chemotaxis of metastatic mammary tumor cells [15,16,18]. In vivo, implanted EGF specifically attracted metastatic tumor cells but not non-metastatic cancer cells in rats [6,7]. Extensive previous work on EGF-induced chemotaxis makes it an ideal system to study the molecular mechanisms involved in cancer cell chemotaxis and their relation to metastasis.

Compared to neutrophils, cancer cells exhibit considerably slower average migration speed (on the order of 1 μm/min compared to 10 μm/min). Therefore, it is critical to

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maintain a stable gradient over an extended period. Effective chemotaxis requires coordination of gradient sensing, polarization and locomotion [19]. The mechanism by which a cell senses and interprets gradients around the cell body is not well understood. A sufficient difference in the concentration of external gradients across the cell is required to activate chemotaxis. Specific gradient, defined as the percentage concentration change per cell length is used to describe the steepness of a gradient across the cell body [20]. Neutrophils, under optimal gradients, can sense a gradient as shallow as 1% difference across the cell body [21]. Such sensitivity may be necessary for the function of highly motile cells such as neutrophils and other leukocytes in the body. For neurons, it has been reported that a minimum of 10% difference in external gradients of neurotransmitters is required to induce turning of growth cones [22]. The sensitivity of cancer cells, especially metastatic ones, to external gradients has not been quantitatively characterized thus far.

Neutrophils adopt a polarized morphology to external attractant gradients without changing the distribution of chemokine receptors on cell surfaces [23]. Similarly, EGF receptors are uniformly distributed on the cancer cell surface [24]. When cancer cells encounter an EGF gradient produced by a micropipette, receptor distribution on the cell surface does not change but the endocytosed receptors are polarized toward the upgradient side of the cell. This receptor internalization is suggested as the first step for EGF signal amplification since the difference of endocytosed EGF receptors across the cell is larger than the difference of external EGF gradients across the cell [24].

We have previously reported that microfluidic chemotaxis chambers (MCC) can generate stable and precise spatial biochemical gradients on the order of microns, and are useful in investigating chemotaxis of neutrophils in chemokine (IL-8) gradients [25,26]. In this study, MCC was used to study the response of human breast cancer cells MDA-MB-231 to different EGF gradient profiles. We first demonstrated the compatibility of MCC with cancer migration by observing enhanced motility of MDA-MB-231 cells in uniform EGF concentrations. We then examined migration in simple linear gradients of EGF. Our data show that linear EGF gradients, regardless of their slopes, are not sufficient to trigger chemotaxis of MDA-MB-231 cells. Rather, MDA-MB-231 cells showed a chemotactic response to non-linear EGF gradient profiles, with an optimum range of 0–50 ng/ml.

Materials and methods

Cell culture

Leibovitz's L-15 medium, penicillin/streptomycin, trypsin/EDTA, cell dissociation buffer, and fetal bovine serum were obtained from Invitrogen (Carlsbad, CA). Recombinant human EGF, bovine serum albumin, and collagen type IV were obtained from Sigma (St Louis, MO). Human metastatic breast cancer cell line MDA-MB-231 was purchased from American Type Culture Collection (Manassas, VA) and cultured in L-15 medium supplemented with 10% FBS and penicillin/streptomycin at 37°C in an air incubator.

Fabrication of microfluidic chemotaxis chambers (MCC)

Embedded networks of microchannels were fabricated in poly(dimethylsiloxane) (PDMS) using rapid prototyping and soft lithography [25]. An irreversible seal was formed between the PDMS replica and glass substrate immediately after air plasma (Harrick Scientific, NY) treatment. This assembly produced the required system of microfluidic channels. The glass surface inside MCC was coated with 2 μg/ml of collagen type IV for 30 min at room temperature and then blocked with 2% BSA for 1 h at 37°C before use. A new MCC was used for each experiment.

Generation of gradients

EGF gradients were generated inside MCC by infusing medium and EGF-containing medium into the two inlets at the top of the chamber (Figs. 3A and 4A). The principle behind the generation of concentration gradient using MCC has been described previously [27]. In this paper, EGF

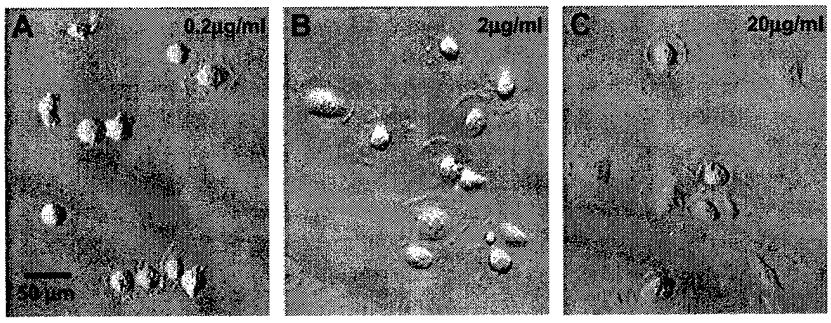

Fig. 1. Effects of collagen type IV coating concentrations on MDA-MB 231 cell migration. Serum-starved MDA-MB-231 cells were loaded into MCC coated with three different concentrations of collagen type IV. Hoffman-modulation-contrast images were taken 1 h after cells attached and spread out.

Table 1
Effect of collagen IV coating concentration on migration parameters

Concentration (μg/ml)	0.2	2	20
Motility (%)	11.86	43.59	15.38
Net cell displacement (μm)	20 ± 2.50	46.83 ± 5.81**	22.36 ± 2.67
Speed (μm/min)	0.69 ± 0.05	0.79 ± 0.04	0.46 ± 0.02
No. of cells analyzed	59	39	39

This table shows the effect of collagen type IV coatings on cell migration. Migration parameters were obtained from time-lapse images of cancer cells migrating in the presence of 25 ng/ml EGF in 0.2% BSA/L15 medium under flow condition (1 μl/min) for 3 h. Motility is calculated as the percentage of cells that exhibited a net cell displacement greater than 25 μm over 90-min periods. The speed for each cell was calculated as the total cell trajectory divided by the migration time. Numbers represent average values ± SE.
** $P < 0.01$.

solutions of appropriate concentrations were prepared in L15 medium with 0.2% BSA. FITC-dextran (10 μM, Mr 10,000), which has a similar molecular weight to EGF (Mr 6,000), was added to the EGF solution as an indicator. The fluorescence intensity profile thus reflects the gradient distribution of EGF inside the channel. The fluorescence micrographs of the FITC-dextran gradient in the migration areas were taken with a digital camera, and the fluorescent intensities were analyzed using a software package, MetaMorph (Universal Imaging, PA). In the polynomial gradient shown in Fig. 4A, there are two distinct regions: the fluorescence intensity remains very low from 0 to approximately 160 μm, but increases rapidly from approximately 160 to 400 μm.

Cell migration inside MCC

MDA-MB-231 cells were serum-starved overnight in 0.2% BSA/L-15 medium before experiments. Cells were detached from the culture dish surface using cell dissociation buffer, washed, and resuspended in 0.2% BSA/L-15 medium. Single-cell suspension was obtained by passing the cell suspension through 40-μm filters (Falcon, BD Labware, Franklin Lakes, NJ). Cells (10–20 μl suspension of 50,000 cell/ml) were loaded into MCC via a cell loading port using a micropipette tip. Continuous flow of medium and growth factor containing medium (1 μl/min) was maintained for the duration of the experiment (1.5–24 h). Cells typically spread out within the first 10 min and started migrating. Time-lapse images were acquired after 10 min of plating the cells. The shear stress subjected to the cancer cells in our experiments was approximately 0.02 dyn/cm^2 (channel dimension of 0.4 × 0.1 × 12 mm and flow rate of 1 μl/min). This is significantly lower than physiological shear stress experienced by endothelial cells (approximately 10 dyn/cm^2).

Time-lapse microscopy and migration data analysis

Microfluidic chemotaxis chambers were placed on an inverted microscope (Nikon TE300) equipped with a heated enclosure at 37°C. Images were acquired every 2 min using a CCD camera, (CoolSNAP cf, Roper Scientific, Duluth, GA) during the experiment (1.5–24 h). Time-lapse images were tracked and analyzed using MetaMorph. Raw tracking data for each cell were analyzed with a spreadsheet to calculate various migration parameters: (1) net cell displacement (straight length of cell displacement between starting and final position), (2) total cell trajectory (sum of straight-line segments a cell travels between 2 successive images), (3) net displacement towards gradient, (4) directionality (ratio of net cell displacement to total cell trajectory), (5) chemotactic index (CI, ratio of net displacement towards

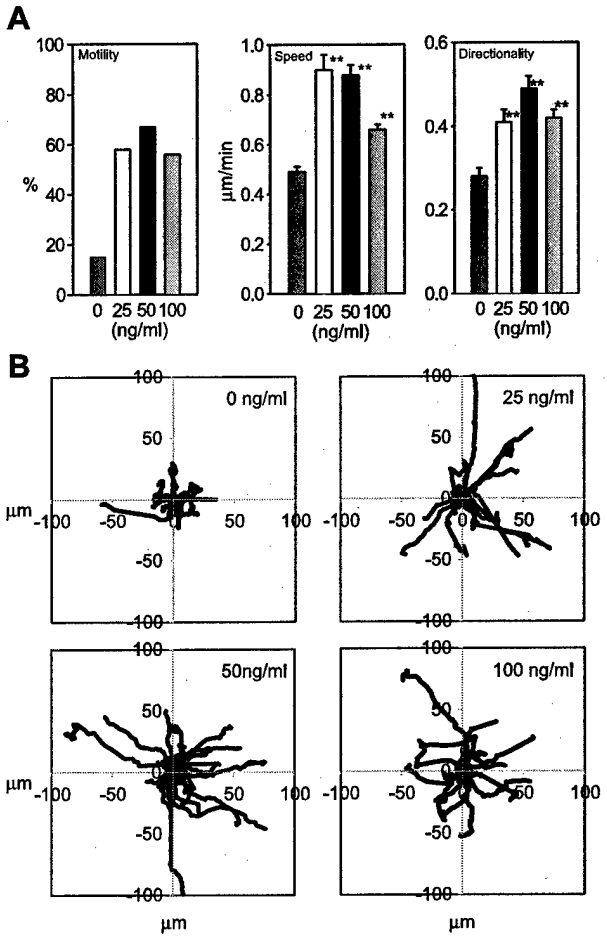

Fig. 2. Migration of MDA-MB-231 cells inside MCC in uniform EGF concentrations. (A) Three migration parameters (motility, speed, and directionality) were calculated for each concentration based on cell migration experiments over 90-min periods. Motility is calculated as the percentage of cells that exhibited a net cell displacement greater than 25 μm. Directionality (ratio of net cell displacement to total cell trajectory) is a measure of how straight the cells move. Cells moving in a straight line have a directionality of 1.0. Bar charts indicate mean ± SE. **$P < 0.01$. The cell numbers analyzed are 87, 65, 75, and 94 for 0, 25, 50, and 100 ng/ml EGF, respectively. (B) Superimposed migration trajectories of cells stimulated with uniform EGF (0, 25, 50, and 100 ng/ml) for 90 min. Initial positions of the cells were superimposed at the origin for comparison. Each plot represents data from 20 cells.

gradient and total cell trajectory), and (6) speed (total cell trajectory divided by time).

Statistical analysis

Student's t test and analysis of variance (ANOVA) were used to determine statistical significance. Results were considered to be significant at $P < 0.05$ or $P < 0.01$.

Supplemental information

Time-lapse images of MDA-MB-231 cell migration in MCC was recorded at 2-min intervals over 3 h. Movie 1 shows MDA-MB-231 cells migrating in a linear, 0 to 50 ng/ml (increasing from left to right) EGF gradient. Image on the right is an overlay of the last frame of this movie with trajectories of 10 randomly selected cells. Movie 2 shows the migration of MDA-MB-231 cells in a nonlinear, 0 to 50 ng/ml (increasing from left to right) EGF gradient.

Results and discussion

Collagen type IV (0.01 μg/ml to 100 μg/ml), Collagen type I (0.05 to 100 μg/ml), vitronectin (0.01 μg/ml to 50 μg/ml), and Matrigel (0.1 to 100 μg/ml) were tested to optimize extracellular matrix (ECM) coatings for migration. At high coating concentrations (100 μg/ml) of ECM, cells attached tightly and remained round, exhibiting poor migration (low speed and small displacement). At low concentrations, below 0.1 μg/ml, cells did not spread out and could not translocate effectively. To characterize the effect of matrix coatings on cell migration, we analyzed 3-h time-lapse images of MDA-MB-231 migrating inside MCC coated with 0.2, 2, and 20 μg/ml of collagen type IV. Cancer cells adhered and spread out on all coated substrates. At high concentrations (2 and 20 μg/ml), cells settled down and spread out faster and exhibited broader lamellipod extension in the presence of 25 ng/ml EGF as compared to the 0.2-μg/ml coating (Figs. 1A, B and C). We quantified the effects of coating on migration parameters by comparing motility (percentage of cells with net cell displacement greater than 25 μm over 90-min periods), net cell displacement, and speed (Table 1). At 0.2 μg/ml collagen type IV, only 12% of cells were motile. The percentage of motile cells increased to 44% when the coating concentration increased to 2 μg/ml, and then dropped to 15% at the 20-μg/ml coating. This result agrees with previous reports [28,29], which showed that motility

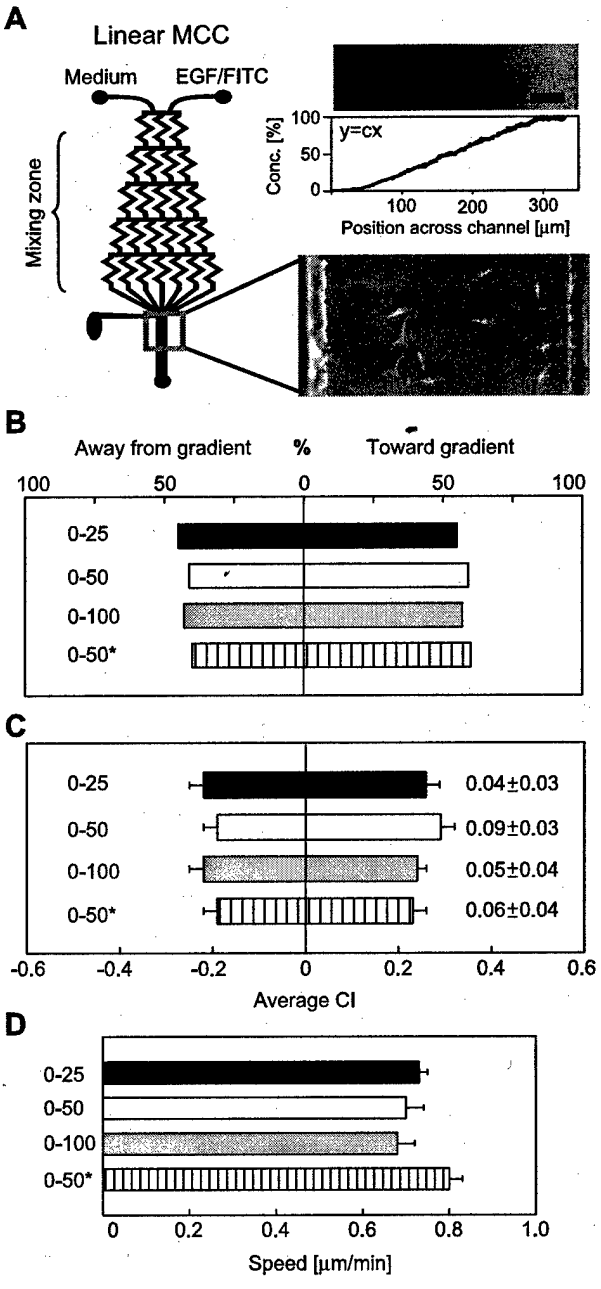

Fig. 3. (A) Design of microfluidic network used to generate linear solution gradients of EGF using MCC. Medium and EGF/FITC solutions were injected into two separate inlets at the top of MCC using syringe pumps (not shown). The fluorescence micrograph shows the solution gradient of FITC-dextran in the cell migration area. The plot below the micrograph shows the corresponding fluorescence intensity profile across the channel. The equation for the linear intensity profile is $y = cx$, where y is the EGF concentration in %, x is the position in channel in μm, and C is a constant. The micrograph shows MDA-MB-231 cells after they spread out inside the migration channel. The scale bar is 50 μm. (B) MDA-MB-231 cells were subjected to linear gradients of EGF: 0–25, 0–50, and 0–100 ng/ml inside MCC with channel width of 350 μm. *Note: 0–50 ng/ml concentration range was tested in a narrower 250 μm channel. Cells were divided into two groups: those that move toward the gradient (with positive CIs) and away from the gradient (with negative CIs) and the percentages (B) and average CI (C) were calculated for each group separately. Average CI of both positive and negative CIs (mean ± SE) is shown next to the bars. (D) Average migration speed was obtained from all cells. The numbers of cells analyzed for each condition are 77, 73, 61, and 77 for 0–25 ng/ml, 0–50 ng/ml, 0–100 ng/ml, and 0–50* ng/ml, respectively.

of cells show biphasic behavior with respect to adhesiveness of the substrate. Cell movement is most effective at 2 μg/ml of coating, as judged by the highest speed and the longest net cell displacement (Table 1). Optimum spreading and migration of MDA-MB-231 cells were observed in chambers coated with 2 μg/ml collagen type IV (30 min at room temperature). All other experiments reported in this work used this ECM coating condition.

Effect of uniform EGF concentrations on MDA-MB-231 cancer cell migration

Fig. 2A shows motility, speed, and directionality of MDA-MB-231 cells as a function of EGF concentration. The percentage of motile cells increased from 15% (no EGF) to as much as 70% when EGF was added to the medium. The average baseline speed of MDA-MB-231 without EGF stimulation was 0.5 μm/min. When EGF was increased to 25 ng/ml or 50 ng/ml, the speed increased to approximately 0.9 μm/min. Further increase in EGF concentration to 100 ng/ml decreased the speed to 0.67 μm/min, higher than the average baseline speed but lower than the average speed of cells in 25 or 50 ng/ml EGF ($P < 0.001$).

Similar to its effect on speed and motility, EGF stimulation enhanced directionality, a measure of how straight the cells move, approximately 1.5-fold. The combined effects of increased speed and directionality resulted in greater displacement away from origin. Fig. 2B shows the migration tracks of 20 representative cells from each condition. Without EGF stimulation, most cells remained within 30 μm from the starting position. In sharp contrast, with EGF stimulation, cells migrated over longer distances and covered wider areas as shown in the trajectories. Most cells displaced greater than 50 μm and some more than 100 μm.

The migration data for MDA-MB-231 cells in uniform concentrations of EGF demonstrate the compatibility of MCC with cancer cell migration. Inside MCC, under constant flow of EGF-containing medium, breast cancer cells moved at speeds comparable to those on other substrates [16]. Cells remained viable and motile for more than 24 h inside MCC under continuous flow of EGF, allowing long-term migration experiments.

MDA-MB-231 breast cancer cell migration in linear EGF gradients

To study the effect of different EGF gradient profiles on cell migration, MDA-MB-231 cells were placed in linear EGF gradients with different slopes. First, three concen-

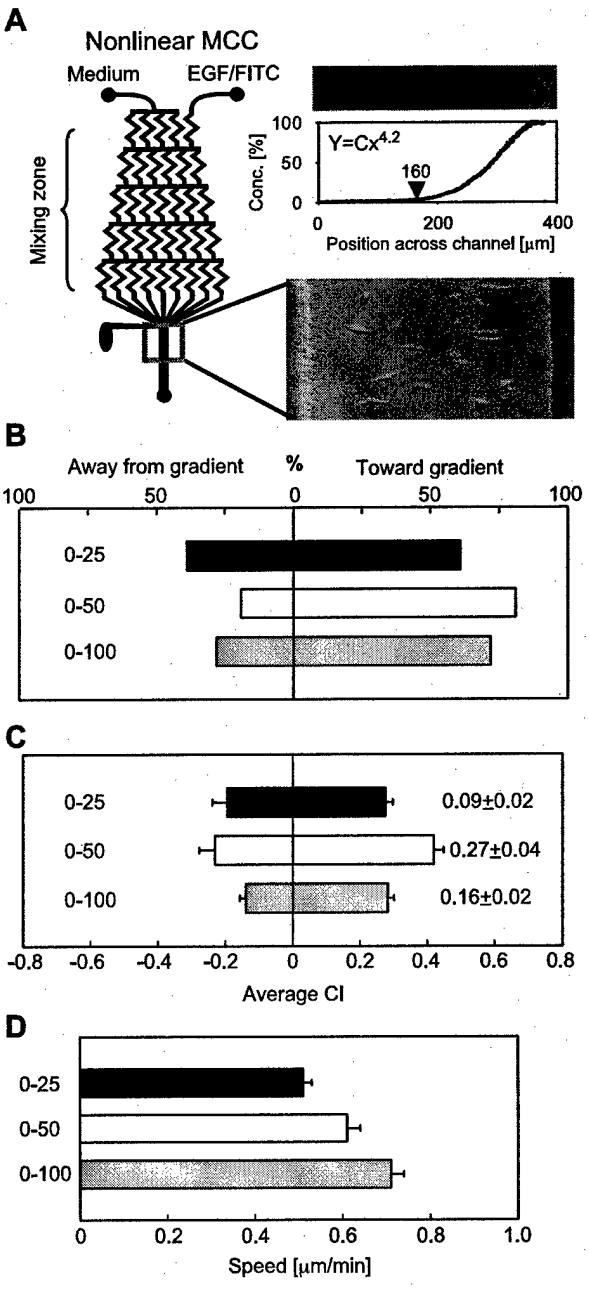

Fig. 4. (A) Design of microfluidic network used to generate nonlinear polynomial solution gradients of EGF. The fluorescence micrograph shows the solution gradient of FITC-dextran in the cell migration area. The plot below shows the corresponding fluorescence intensity profile across the channel. The nonlinear equation for the intensity profile is $y = Cx^{4.2}$, where y is the EGF concentration in %, x is the position in channel in μm, and C is a constant. The micrograph shows MDA-MB-231 cells after they spread out inside the migration channel and subjected to nonlinear EGF gradient. The scale bar is 50 μm. MDA-MB-231 cells were subjected to three nonlinear EGF gradients: 0–25, 0–50, and 0–100 ng/ml inside MCC (channel width = 400 μm). Cells were divided into two groups: those that move toward the gradient (with positive CIs) and away from the gradient (with negative CIs) and the percentages (B) and average CI (C) were calculated for each group separately. Average CI of both positive and negative CIs (mean ± SE) is shown next to the bars. (D) The average migration speed was obtained from all cells. The numbers of cells analyzed for each concentration range are 110, 89, and 159 for 0–25 ng/ml, 0–50 ng/ml and 0–100 ng/ml, respectively.

tration ranges (0–25, 0–50, and 0–100 ng/ml) with slopes of 71, 143, and 286 pg $ml^{-1}\mu m^{-1}$, respectively, across 350 μm wide migration channels, were tested. Fig. 3A shows the design of the microfluidic network that produced linear gradients used for the experiment. This type of MCC has been previously used to investigate neutrophil migration in linear IL-8 gradients [25,26]. The fluorescence micrograph and the fluorescence profile plot (Fig. 3A, upper two panels) show a linear fluorescence intensity profile due to FITC-Dextran gradient. This gradient in fluorescence indirectly verifies the gradient of EGF which was introduced into MCC through the same inlet (see Material and methods for details).

Fig. 3B shows the percentages of cells that moved toward gradient and away from gradient; Fig. 3C shows the average chemotactic index (CI) of cells that moved in either direction separately. CI is a measure of how much of the total movement is directed toward the gradient, ranging from −1 (cell moves completely away from gradient direction) to 1 (cell moves completely toward gradient direction). In all linear gradients, approximately 60% of the cells moved toward the gradient while the other 40% moved away from the gradient (Fig. 3B, also see Movie 1 in supplementary material). The combined average CI values (average of positive and negative CI values, shown to the right of each bar) were less than 0.1 for all conditions, suggesting that there was no obvious directed movement toward higher concentrations of EGF in these linear gradients.

To test whether a higher slope with the same concentration range (i.e., 0–50 ng/ml) would induce effective chemotaxis, the migration channel width was reduced from 350 to 250 μm. With a narrower channel, a linear gradient with a slope of 200 pg $ml^{-1}\mu m^{-1}$ was generated. Cells continued to migrate in a random manner similar to the other linear gradients. No obvious chemotactic response was observed as indicated by the percentage (60%) of cells that moved toward the gradient (Fig. 3B, 0–50*) and the average CI (0.06) (Fig. 3C, 0–50*).

Breast cancer cell migration in nonlinear EGF gradients

Random migration of cancer cells under EGF gradients contrasts with recent reports of MDA-MB-231 [12,13] and metastatic rat mammary adenocarcinoma (MTLn3) [7,16,17] chemotaxis toward EGF. These reports used Boyden chambers and micropipette-based assays to test chemotaxis. These assays generate nonlinear gradient profiles because they are based only on molecular diffusion. Thus, we modified the design of MCC to generate a nonlinear, polynomial gradient that has the form $y = Cx^{4.2}$ [30]. Fig. 4A shows the design of MCC and the profile of the gradient produced using this device. This nonlinear gradient profile is similar to the early stage of IL-8 and

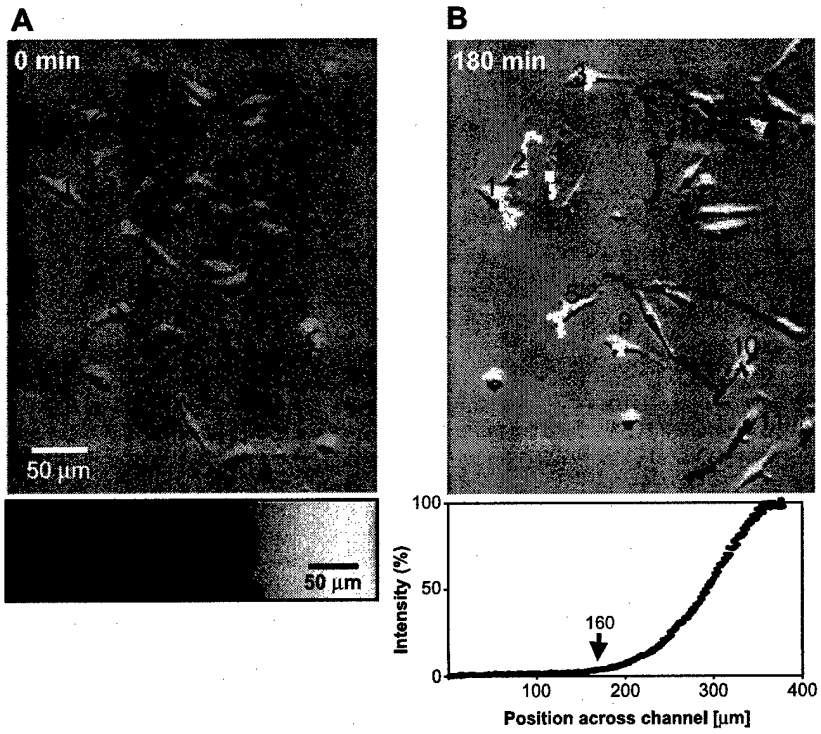

Fig. 5. Chemotaxis of MDA-MB-231 cells in a nonlinear gradient of EGF. First (A) and last (B) frames of a 3-h time-lapse acquisition of cells migrating in a nonlinear EGF gradient, 0–50 ng/ml. (B) Cells that moved toward higher EGF concentration were marked with black trajectories, while cells that moved away from the EGF gradient were marked with white. The fluorescence micrograph (A, lower panel) shows the solution gradient of FITC-dextran in the cell migration area. A plot of the gradient profile (B, lower panel) shows the corresponding fluorescence intensity across the channel.

LTB4 gradients produced in under agarose assays [31] and the neurotransmitters gradient profiles generated by repetitive pulsatile ejection from micropipettes [32].

The same EGF concentration ranges (0–25, 0–50, and 0–100 ng/ml) that were used in the linear gradients were tested with the nonlinear MCC. All of the three nonlinear gradients tested resulted in higher percentages of cells moving toward gradients, at least 61% (in 0–25 ng/ml) and as high as 81% (in 0–50 ng/ml) (Fig. 4B). Significant chemotactic responses to EGF gradients were observed in two nonlinear EGF gradients: 0–50 ng/ml (81% toward gradient, CI = 0.27, see Movie 2 in supplementary material) and 0–100 ng/ml (71% toward gradient, CI = 0.16, Figs. 4B and C). The 0–50 ng/ml EGF nonlinear gradient was the optimum condition, not only attracting the highest percentage of cells toward gradient, but also exhibiting the highest average CI among the three ranges tested.

Subregion analysis

Fig. 5 shows the first and last frames of a time-lapse recording of cells moving in a 0–50 ng/ml EGF nonlinear gradient over a 3-h period. In the first frame, cells started to extend lamellipods and displayed polarized morphology. In the final frame (after 180 min), cells that moved with positive CI values (chemotactic to EGF) were marked in black and cells with negative CI values (not chemotactic to EGF), were marked in white. Majority of cells on the left side of the chamber were not polarized or randomly polarized and thus did not exhibit significant displacement toward the gradient (Fig. 5B). In sharp contrast, most of the cells on the right showed lamellipod extension and polarization toward gradient and exhibited long displacement toward the gradient. Detailed examination of the nonlinear gradient profile (lower panels of Fig. 5) and cell migration trajectories revealed that cells in nonlinear gradients can be divided into two distinct groups based on their positions in the gradient. We thus divide the gradient profile into a nonlinear shallow concentration region (0 to 160 μm) on the left side of channel, where EGF concentration increases slowly from 0 to 0.2 ng/ml, and a nonlinear steep region (160–400 μm) on the right, where the concentration increases rapidly from 0.2 to 50 ng/ml. Cells were then classified and analyzed separately based on their final positions in the shallow or steep regions.

At optimum range of nonlinear EGF gradient, 0–50 ng/ml, the drastic difference in migration behavior of cells in the two regions is clearly reflected by the trajectories (Fig. 6A). Cells in the shallow region (left) moved in random patterns and exhibited shorter paths as compared to cells in the steep region (right), which exhibited a strong directional preference toward the higher EGF concentrations. Cells were more motile in the steep region and showed longer paths. In the 0–100 ng/ml EGF nonlinear gradient, 75% (Fig. 6B upper left panel) of cells in the steep region moved toward the gradient with an average speed of 0.77 μm/min (lower left panel). This speed was higher than that in 0–50 ng/ml, in which cells moved at 0.62 μm/min ($P < 0.01$) (Fig. 6B lower left panel). However, cells seemed meander more, which resulted in lower directionality than that in 0–50 ng/ml (0.36 compared to 0.45, $P = 0.02$) (lower right panel). The average CI of 0–100 ng/ml was lower than that of 0–50 ng/ml (0.20 compared to 0.32, $P < 0.05$) in the steep region (Fig. 6B upper right panel).

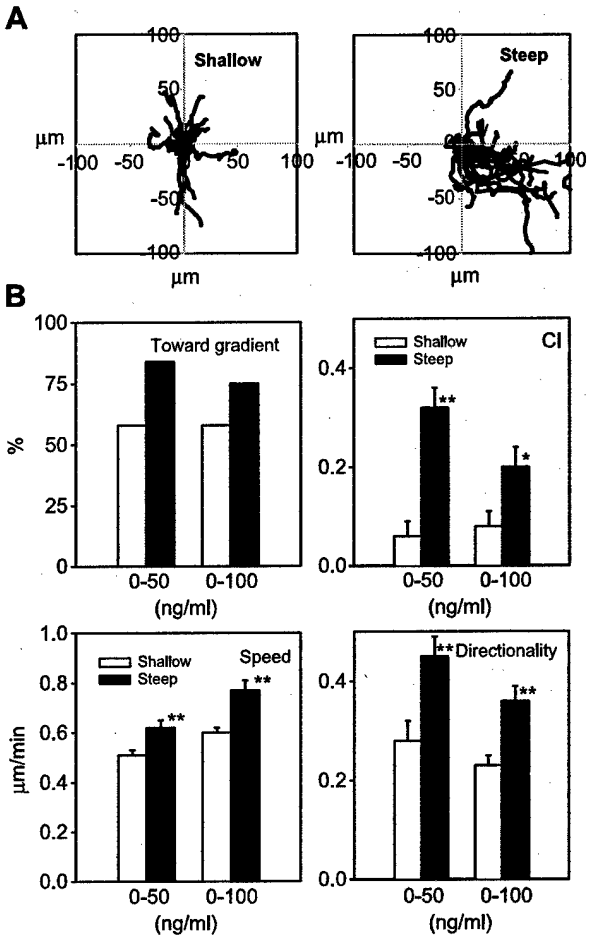

Fig. 6. Subregion analysis of the directional movement of MDA-MB-231 cells in nonlinear EGF gradients. The nonlinear gradient was divided into two regions, a 160-μm wide flat, shallow region on the left, and a 240-μm wide steep region on the right. The cells were grouped according to their final positions. (A) Superimposed trajectories for cells in the shallow (27 cells) and steep (20 cells) region of 0–50 ng/ml nonlinear EGF gradients. (B) Comparison of average migration parameters for the 0–50 and 0–100 ng/ml nonlinear EGF gradients after subregion analysis. Each bar chart represents mean ± SE. $^{**}P < 0.01$ and $^{*}P < 0.05$. Total of 52 and 93 cells were analyzed for the shallow and steep region for 0–50 nonlinear gradients. Total of 60 and 100 cells were analyzed for the shallow and steep region for 0–100 nonlinear gradients.

The chemotactic response of breast cancer cells to EGF gradients became more apparent when the cells in the steep region of the nonlinear gradients were analyzed separately from those in the shallow region. Low concentrations of EGF (0–0.2 ng/ml over 160 μm) in the shallow region might fail to activate a cell to move directionally toward

gradient. On the other hand, cells in the steep region experience much higher concentrations of EGF (0.2 to 50 ng/ml over 240 μm). This range, according to our data, is optimum for chemotaxis as it induced the highest percentage of cells moving toward the gradient (84%) and the highest average CI (0.32) (Fig. 6B). Although cells moved faster when the EGF concentration range was increased to 100 ng/ml (Fig. 6B), the overall chemotactic response decreased, as evidenced by lower percentage of cells that moved toward gradient, lower average CI, and less directionality (Fig. 6B). This suggests that in addition to gradient profiles, the EGF concentration range also affects the effectiveness of chemotaxis.

Inside MCC, a cell's front and rear ends sense different concentrations of EGF (Fig. 7A). The specific gradient ($[\Delta C/C_m]/50$ μm, in Fig. 7B), defined as the percentage concentration change over one cell length (50 μm), is calculated as the concentration difference between the front and back (ΔC), normalized by the EGF concentration at the middle of the cell (C_m). At all positions inside MCC, cells in nonlinear gradients exhibit higher specific gradients than cells in linear gradients (Fig. 7B). Although the two specific gradients appear to approach each other at about 160 μm from the left, the specific gradients in nonlinear MCC are still four times higher than those in linear MCC. This suggests that there might be a threshold value for the specific gradient of EGF to induce effective chemotaxis of MDA-MB-231 cells. Cells in linear gradients might experience sub-threshold specific gradient and thus failed to move toward EGF gradients.

Moreover, in addition to specific gradient surrounding cells, interaction of EGF with EGF receptor (EGFR) during chemotaxis needs to be considered. In a simple receptor-ligand binding model, fraction of receptors occupied (FRO) by ligand EGF is calculated, $FRO = C/(C + K_d)$, where K_d (equilibrium dissociation constant) ≅ 1 nM for EGFR [24]. Fig. 7C shows that in the linear gradient of EGF (0–50 ng/ml, grey curve) cells at the low end of the gradient exhibit an FRO of about 0.23, which increases rapidly to 0.7 as the cell moves to position 90 μm. The FRO then increases gradually and reaches plateaus (approximately 0.87) at position 270 μm. In contrast, the FRO of cells in the nonlinear EGF gradient of the same range (Fig. 7C, black curve) increases slowly at the low end of the nonlinear gradient. A sharp increase to approximately 0.89 in FRO is seen when the cell move past 160 μm toward the high end of the gradient. DFRO (the calculated difference of FRO between the front and the rear of a cell in the gradient), a measure of the strength of the chemotactic signal to the cell [33,34], is shown in Fig. 7D. In linear gradients, DFRO

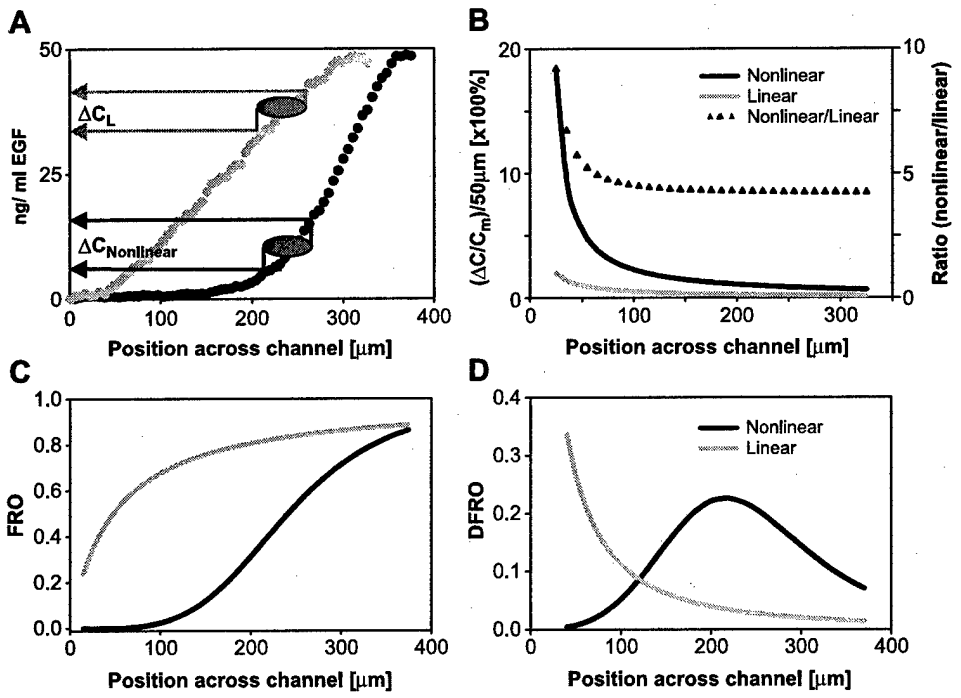

Fig. 7. (A) The front and rear end of a cell sense different concentrations of EGF as it migrates across an EGF gradient. The concentration difference (?C) between the front and rear depends on the gradient profile (nonlinear vs. linear) and the position of the cell inside the channel. The specific gradient ($\Delta C/C_m$ over a cell length of 50 μm) in (B) is defined as the concentration difference between front and rear (ΔC) normalized by the EGF concentration at the middle of the cell (C_m). Solid triangles (scaled from 0 to 10 on the right) in B show the ratio of the two specific gradients (nonlinear/linear). (C) Calculated fractional receptor occupancy ($FRO = C/(C + K_d)$, K_d ≅ 1 nM for EGFR) of cells in linear (grey) and nonlinear (black) 0–50 ng/ml EGF gradient. (D) Calculated difference in fractional receptor occupancy (DFRO) of EGF receptors between the front and rear of cells moving in 0–50 ng/ml linear (grey) and nonlinear (black) EGF gradients. The size of the cell was assumed to be 50 μm in length.

decreases rapidly from 0.34 to 0.01 across the channel. In contrast, DFRO in the nonlinear EGF gradient is a bell-shaped curve with its highest value around the middle of the channel. To the right of the 100 μm position, cells in nonlinear gradients experience higher DFRO than cells in linear gradients, and this could explain why cells chemotax more effectively in nonlinear EGF.

We have developed and implemented MCC for the investigation of metastatic breast cancer cell migration in response to concentration gradients of EGF. We analyzed the movement patterns of individual cells in different gradients and quantified the extent to which the movement is directed (chemotactic) in each gradient. We found that the migratory response of cells depends not only on the gradient profile, but also on the range of EGF concentrations. In all linear gradients with different steepness and mean concentrations tested, cells moved randomly and the average chemotactic index (CI) did not exceed 0.1. The same ranges induced directed overall movement when the gradient took on a nonlinear profile. In this case, optimum chemotaxis was observed in 0–50 ng/ml EGF, with an average CI of 0.27.

Using MCC, the migration paths of individual cells can be studied in detail and under well-controlled conditions that can be manipulated with great flexibility. Such control is not possible in traditional chemotaxis assays, which usually suffer from being endpoint assays and/or are unable to maintain stable gradients. Moreover, the migratory patterns in our assay can be tracked for several cells at a time, providing sample sizes that are statistically meaningful. This is in contrast with micropipette diffusion assays, which are low throughput and rely on single-cell tracking. Using MCC, we can gain a better quantitative understanding of cancer cell chemotaxis. Since chemotaxis is an important step in the metastatic cascade, a better biological mechanistic understanding and quantitative analysis of the process should provide valuable insights and guide for future therapeutic approaches.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yexcr.2004.06.030.

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